

Synthesis and Fluorogenic Properties of Some 1-(Coumarin-7-yl)-4,5-dihydro-1,2,4-triazin-6(1*H*)-ones

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Abstract

Herein, we have synthesized and tested a selected set of N1-(coumarin-7-yl)-4,5-dihydro-1,2,4-triazin-6(1*H*)-ones (**8**) incorporating masked α -amino acids within the triazinone skeleton as synthetic fluorogenic substrates for amino peptidases using human nasal epithelium that expressed aminopeptidases. These chiral triazinones, obtained *via* direct interaction of α -amino esters with N1-(coumarin-7-yl) nitrile imine (generated *in situ* from its hydrazonoyl chloride by the action of triethylamine), bear close resemblance to 7-amino-4-methylcoumarin (AMC) with labeled α -amino acids (AA-AMC's/7). The aminopeptidase activity of the synthesized compounds (0.125 and 0.5 mM) was compared with that of a model compound L-alanine-4-methylcoumaryl-7-amide (Ala-MCA), following in-house validation of the aminopeptidase activities of the human nasal primary culture. In comparison to the model aminopeptidase substrate (Ala-MCA), the synthesized compounds yielded fluorescent 7-amino-4-methylcoumarin following incubation with nasal epithelial cells. However, the yield was significantly lower than was observed for the control compound.

Keywords: 7-Aminocoumarins, L- α -amino esters, Aminopeptidases, N-(coumarin-7-yl)hydrazonoyl chloride, Substituted dihydro-1,2,4-triazin-6-ones

1. Introduction

A number of 1,2,4-triazinone derivatives possess significant biological activities, such as antimicrobial, fungicide, pesticide, herbicide and crop protection agents, as well as blood platelet aggregation-inhibition (Neunhoeffler et al., 1978; El Ashry et al., 1994; Abdel-Rahman, 2001), while some others exhibit anti-HIV and antitumoral activity against leukemia, ovarian cancer, small and large lung cancer cells, and breast cancer (Abdel-Rahman et al., 1999; El-Gendy et al., 2001; Krauth et al., 2010). Among the different 1, 2, 4-triazinones described in the literature, only a few are 4,5-dihydro-1,2,4-triazin-6-ones **1-4** (Fig.1) (El-Abadelah et al., 1991; Dalloul et al., 2008; Collins et al., 1996; El-Abadelah et al., 1997). These compounds are best prepared *via* cyclocondensation reactions of various nitrileimines with the respective α -amino esters [products **1** (El-Abadelah et al., 1991), **2** (Dalloul et al., 2008), **3** (Collins et al., 1996)], or with α -hydrazino esters (products **4**) (El-Abadelah et al., 1997). Other synthetic routes involve cyclization of α -amino acid hydrazide with acetals (Neunhoeffler et al., 1992), cyclization of amidrazones with α -haloesters (Dalloul et al., 2007), and by reaction of hydrazine with *N*-thioacyl α -amino esters (Collins et al., 1996, Andersen et al., 1983), with α -amino acid imidates (Collins et al., 2000) or with methyl α -(dimethylaminomethyleneamino)-carboxylates (amidines) (Smodis et al., 1990; Taylor et al., 1985).

On the other hand, coumarins (also named 2-oxo-2*H*-chromenes or 2*H*-1-benzopyran-2-ones) occur naturally as secondary metabolites in several plant species, and many exhibit useful and diverse biological activities (Egan et al., 1990; Borges et al., 2005). Examples include umbelliferone (**5**) and psoralen (**6**) (Fig. 2). Coumarin derivatives were found to have numerous therapeutic applications such as photochemotherapy (Conforti et al., 2009), antitumor (Harvey et al., 1988; Kostova, 2005; Musa et al., 2008) and anti-HIV therapy (Kostova et al., 2006), as antibacterials (Al-Haiza et al., 2003; Musiciki et al., 2000), anti-inflammatory (Fylaktakidou et al., 2004), anti-coagulants (Jung et al., 2001) and dyes (Wang et al., 2005).

Interestingly, 7-amino-4-methylcoumarin (AMC) (Pozdnev, 1990) is employed for labeling L- α -amino acids (AA) *via* coupling of the respective AA-carboxyl and AMC-7-amino functions. These labeled α -amino acids (AA-AMC's/7) (Fig. 3) have been utilized as synthetic fluorogenic and highly specific substrates for aminopeptidases, in which case hydrolysis of the amide bond results in liberation of the fluorescent AMC (Iwaki et al., 1986; Acosta et al., 2008; Budić et al., 2009; Semashko et al., 2008; Chung, 2008; Townsend, 2009). As a result, such substrates offer rapid detection, visualization and assay of various aminopeptidase enzymes from different / target microorganisms in samples or plant extracts (Iwaki et al., 1986; Acosta et al., 2008; Budić et al., 2009; Semashko et al., 2008; Chung, 2008; Townsend, 2009).

In this context, we sought to prepare a selected set of 1-(chromen-7-yl)-4,5-dihydro-1,2,4-triazin-6(1*H*)-ones incorporating masked α -amino acid residues within the triazinone ring system, as exemplified by **8** (Fig. 3). Their synthetic route and structural varieties are illustrated in Schemes 1 and 2 (given in the results part). These derivatives (**8**) bear close resemblance to their AA-AMC's counter parts (**7**), shown in Fig. 3, and thus might act as fluorogenic substrates for aminopeptidases enzymes in a similar manner to **7**.

2. Experimental

2.1 Materials and equipments

The following chemicals used, in this study, were purchased from Acros and were used as received: (L)- α -Amino acid methyl esters of alanine, leucine, phenylalanine, glycine, tryptophane, proline, methionine, sarcosine, serine and threonine), (L)- α -amnio acid dimethyl esters of aspartic and glutamic acids. L-alanine-4-methylcoumaryl-7-amide (Ala-MCA), 7-Amino-4-methylcoumarin (AMC), protease type XIV (pronase) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Silica gel for column chromatography was purchased from Macherey-Nagel GmbH & Co (Germany). Phenol red, Phosphate buffered saline and Hanks' balanced salt (HBSS), TriZol, M-MVL reverse transcriptase, cDNA buffer, dNTPs and dNTP were supplied by Invitrogen (Carlsbad, CA, USA). MgCl₂, Taq PCR buffer, and Taq polymerase were provided by Fermentas (Burlington, ON, USA). Agarose powder and oligo dT primers were from Fisher Scientific (Ottawa, ON, Canada) and Promega (Madison WI, USA), respectively.

Melting points (uncorrected) were determined on a Stuart scientific melting point apparatus in open capillary tubes. Optical rotations were taken on a Perkin Elmer 141 photoelectric spectropolarimeter in dimethylformamide ($c \sim 1$), at 20 ± 1 °C. ¹H- and ¹³C-NMR spectra were recorded on a 300 MHz spectrometer (Bruker DPX-300) with TMS as the internal standard. Chemical shifts are expressed in δ units, J -values for ¹H-¹H coupling constants are given in Hertz. High resolution mass spectra (HRMS) were acquired (in positive / or negative mode) using electrospray ion trap (ESI) technique by collision-induced dissociation on a Bruker APEX-4 (7-Tesla) instrument. The samples were dissolved in acetonitrile, diluted in spray solution (methanol/water 1:1 v/v +0.1% formic acid) and infused using a syringe pump with a flow rate of 2 μ L/min. External calibration was conducted using arginine cluster in a mass range m/z 175-871.

2.2 7-Amino-4-methylcoumarin (**9**)

This synthon, required in the present study, was prepared from *m*-aminophenol by following a literature procedure (Pozdnev, 1990).

2.3 *N*-(4-Methyl-2-oxo-2*H*-chromen-7-yl)-2-oxo-propanehydrazonoyl chloride (**10**)

Compound **9** (17.5 g, 0.10 mol) was dissolved in 17% aqueous hydrochloric acid (160 mL) and cooled to 0 °C. To this solution was added, dropwise, a solution of sodium nitrite (7.6 g, 0.11 mol) in water (15 mL) with efficient stirring at 0 - 5 °C. Stirring was continued for 20 - 30 min, and the resulting fresh cold 4-methyl-2-oxo-2*H*-chromene-7-diazonium chloride solution was poured onto a cold solution (0 to -10 °C, ice-salt bath) of 3-chloropentan-2,4-dione (13.5 g, 0.1 mol) in pyridine / water (160 mL, 3:2 v/v) with vigorous stirring. The resulting orange-colored mixture was further stirred until a solid precipitate was formed (5 - 10 min). The reaction mixture was then diluted with cold water (200 mL), the solid product was collected by suction filtration, washed several times with cold water, dried, and recrystallized from acetonitrile. Yield = 22.8

g (82%), m.p. 271 – 274 °C. ¹H NMR (300 MHz, DMSO-d₆) δ: 2.34 (d, *J* = 1.0 Hz, 3H, CH₃-4), 2.46 (s, 3H, O=C-CH₃), 6.17 (d, *J* = 1.0 Hz, 1H, H-3), 7.27 (d, *J* = 1.8 Hz, 1H, H-8), 7.38 (dd, *J* = 8.7, 1.8 Hz, 1H, H-6), 7.67 (d, *J* = 8.7 Hz, 1H, H-5), 10.97 (s, 1H, N-H). ¹³C NMR (75 MHz, DMSO-d₆) δ: 18.6 (CH₃-4), 26.0 (O=C-CH₃), 101.9 (C-8), 111.8 (C-6), 112.1 (C-3), 115.1 (C-4a), 127.1 (C-5), 146.0 (C-4), 146.2 (C-7), 153.8 (-C=N), 154.9 (C-8a), 160.5 (C-2), 188.6 (O=C-Me). Anal. Calcd for C₁₃H₁₁ClN₂O₃ (278.69 g/mol): C 56.03, H 3.98, Cl 12.72, N 10.05, found: C 56.12, H 3.91, Cl 12.58, N 9.92. HRMS(ESI) *m/z*: Calcd for C₁₃H₁₂ClN₂O₃ [M + H]⁺ 279.05364, found 279.05310.

2.4 General procedure for the synthesis compounds (8a-k)

To a cold suspension (0 to -10 °C) of 1.80 mmol (0.5 g) compound (10) in 20 mL of ethanol was added, with stirring, a solution of appropriate L-(α)-amino acid methyl esters (2.0 mmol) and triethylamine (3 mL) in 10 mL of ethanol. Stirring was continued at 0 to 5 °C for 2 - 4 h, and then at ambient temperature for 10 - 12 h. The solvent was then removed under reduce pressure and the residue was treated with water (15 mL). The resulting crude solid product was collected by suction filtration, washed with water, dried and purified on preparative silica gel TLC plates.

2.4.1 3-Acetyl-1-(4-methyl-2-oxo-2H-chromen-7-yl)-4,5-dihydro-1,2,4-triazin-6(1H)-one (8a)

Yield = 0.42 g (89%), m.p. 282 – 284 °C. ¹H NMR (300 MHz, CDCl₃) δ: 2.40 (br s, 3H, CH₃-4'), 2.46 (s, 3H, O=C-CH₃), 4.00 (s, 2H, H₂-5), 6.32 (br s, 1H, H-3'), 7.65 (d, *J* = 8.7 Hz, 1H, H-5'), 7.71 (br s, 1H, H-8'), 7.75 (br d, *J* = 8.7 Hz, 1H, H-6'). ¹³C NMR (75 MHz, CDCl₃) δ: 18.8 (CH₃-4'), 24.7 (O=C-CH₃), 43.7 (C-5), 112.2 (C-8'), 114.2 (C-3'), 117.8 (C-4'a), 119.8 (C-6'), 125.7 (C-5'), 143.6 (C-4'), 143.7 (C-7'), 153.3 (C-3), 153.4 (C-8'a), 160.3 (C-2'), 160.6 (C-6), 193.0 (O=C-Me). Anal. Calcd for C₁₅H₁₃N₃O₄ (299.28 g/mol): C 60.20, H 4.38, N 14.04, found: C 60.05, H 4.33, N 13.92. HRMS(ESI) *m/z*: Calcd for C₁₅H₁₃N₃NaO₄ [M + Na]⁺ 322.08038, found 322.08007.

2.4.2 (S)-3-Acetyl-5-methyl-1-(4-methyl-2-oxo-2H-chromen-7-yl)-4,5-dihydro-1,2,4-triazin-6(1H)-one (8b)

Yield = 0.48 g (84%), m.p. 208 – 210 °C, [α]_D -126 (*c* ~ 1, DMF). ¹H NMR (300 MHz, CDCl₃) δ: 1.50 (d, *J* = 6.7 Hz, 3H, CH₃(CH)-5), 2.41 (d, *J* = 1.0 Hz, 3H, CH₃-4'), 2.48 (s, 3H, O=C-CH₃), 4.26 (q, *J* = 6.7 Hz, 2H, (Me)CH-5), 5.97 (s, 1H, NH-4), 6.24 (d, *J* = 1.0 Hz, 1H, H-3'), 7.57 (d, *J* = 8.7 Hz, 1H, H-5'), 7.67 (d, *J* = 2.0 Hz, 1H, H-8'), 7.70 (dd, *J* = 8.7, 2.0 Hz, 1H, H-6'). ¹³C NMR (75 MHz, CDCl₃) δ: 19.6 (CH₃(CH)-5), 18.7 (CH₃-4), 25.9 (O=C-CH₃), 49.6 (C-5), 111.9 (C-8'), 114.3 (C-3'), 118.0 (C-4'a), 119.7 (C-6'), 124.4 (C-5'), 142.3 (C-4'), 143.4 (C-7'), 152.1 (C-3), 153.6 (C-8'a), 161.0 (C-2'), 162.9 (C-6), 192.8 (O=C-Me). Anal. Calcd for C₁₆H₁₅N₃O₄ (313.31 g/mol): C 61.34, H 4.83, N 13.41, found: C 61.15, H 4.77, N 13.28. HRMS(ESI) *m/z*: Calcd for C₁₆H₁₅N₃NaO₄ [M + Na]⁺ 336.09603, found 336.09569.

2.4.3 (S)-3-Acetyl-5-isobutyl-1-(4-methyl-2-oxo-2H-chromen-7-yl)-4,5-dihydro-1,2,4-triazin-6(1H)-one (8c)

Yield = 0.40 g (63%), m.p. 164 – 166 °C. [α]_D -320 (*c* ~ 1, DMF). ¹H NMR (300 MHz, CDCl₃) δ: 0.97 (dd, *J* = 6.2, 1.4 Hz, 6H, (CH₃)₂CH), 1.67 (m, 1H, CHMe₂), 1.80 (m, 2H, CH₂-5), 2.43 (d, *J* = 1.0 Hz, 3H, CH₃-4'), 2.46 (s, 3H, O=C-CH₃), 4.18 (m, 1H, H-5), 5.94 (s, 1H, NH-4), 6.26 (d, *J* = 1.0 Hz, 1H, H-3'), 7.59 (d, *J* = 8.7 Hz, 1H, H-5'), 7.69 (d, *J* = 2.0 Hz, 1H, H-8'), 7.72 (dd, *J* = 8.7, 2.0 Hz, 1H, H-6'). ¹³C NMR (75 MHz, CDCl₃) δ: 18.7 (CH₃-4'), 21.5 ((CH₃)₂CH), 23.0 (CH-Me₂), 23.9 (O=C-CH₃), 41.9 (CH₂-5), 52.1 (C-5), 111.8 (C-8'), 114.7 (C-3'), 118.0 (C-4'a), 119.7 (C-6'), 124.4 (C-5'), 142.0 (C-4'), 143.5 (C-7'), 152.0 (C-3), 153.7 (C-8'a), 160.9 (C-6), 162.6 (C-2'), 192.8 (O=C-Me). Anal. Calcd for C₁₉H₂₁N₃O₄ (355.39 g/mol): C 64.21, H 5.96, N 11.82, found: C 64.06, H 5.90, N 11.76. HRMS(ESI) *m/z*: Calcd for C₁₉H₂₂N₃O₄ [M + H]⁺ 356.16103, found 356.16048.

2.4.4 (S)-3-Acetyl-5-benzyl-1-(4-methyl-2-oxo-2H-chromen-7-yl)-4,5-dihydro-1,2,4-triazin-6(1H)-one (8d)

Yield = 0.38 g (54%), m.p. 167 – 171 °C. [α]_D -438 (*c* ~ 1, DMF). ¹H NMR (300 MHz, CDCl₃) δ: 2.34 (d, *J* = 1.0 Hz, 3H, CH₃-4'), 2.44 (s, 3H, O=C-CH₃), 3.10 (m, 2H, -CH₂Ph), 4.47 (m, 1H, H-5), 5.88 (s, 1H, NH-4), 6.27 (d, *J* = 1.0 Hz, 1H, H-3'), 7.10-7.14 (m, 2H, H-2" + H-6"), 7.27-7.30 (m, 3H, H-3" + H-4" + H-5"), 7.55 (d, *J* = 2.0 Hz, 1H, H-8'), 7.59 (d, *J* = 8.7 Hz, 1H, H-5'), 7.63 (dd, *J* = 8.7, 2.0 Hz, 1H, H-6'). ¹³C NMR (75 MHz, CDCl₃) δ: 18.8 (CH₃-4'), 23.8 (O=C-CH₃), 40.3 (CH₂-Ph), 55.2 (C-5), 112.1 (C-8'), 114.8 (C-3'), 118.1 (C-4'a), 120.0 (C-6'), 124.4 (C-5'), 127.7 (C-4"), 129.0 (C-2" / C-6"), 129.6 (C-3" / C-5"), 134.9 (C-1"), 141.9 (C-4'), 143.2 (C-7'), 152.0 (C-3), 153.6 (C-8'a), 160.9 (C-2'), 161.8 (C-6), 192.5 (O=C-Me). Anal. Calcd for C₂₂H₁₉N₃O₄ (389.40 g/mol): C 67.86, H 4.92, N 10.79, found: C 67.94, H 4.84, N 10.57. HRMS(ESI) *m/z*: Calcd for C₂₂H₁₉N₃NaO₄ [M + Na]⁺ 412.12733, found 412.12678.

2.4.5 (S)-3-acetyl-5-(hydroxymethyl)-1-(4-methyl-2-oxo-2H-chromen-7-yl)-4,5-dihydro-1,2,4-triazin-6(1H)-one (**8e**)

Yield = 0.50 g (81%), m.p. 182 – 183 °C. $[\alpha]_D^{25}$ -248 ($c \sim 1$, DMF). $^1\text{H NMR}$ (300 MHz, DMSO- d_6) δ : 2.39 (br s, 3H, CH_3 -4'), 2.40 (s, 3H, $\text{O}=\text{C}-\text{CH}_3$), 3.37 (s, 1H, OH), 3.67 (dd, 2H, CH_2 -5), 4.13 (t, $J = 2.4$, 1H, H-5), 5.16 (s, 1H, NH-4), 6.33 (d, $J = 1.0$, 1H, H-3'), 7.65 (d, $J = 2.0$ Hz, 1H, H-8'), 7.69 (dd, $J = 8.7$ Hz, 2.0, 1H, H-6'), 7.76 (d, $J = 8.7$ Hz, 1H, H-5'). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ : 18.6 (CH_3 -4'), 24.7 ($\text{O}=\text{C}-\text{CH}_3$), 56.5 (CH_2 -5), 63.7 (C-5), 111.3 (C-8'), 114.2 (C-3'), 117.7 (C-4'a), 119.9 (C-6'), 125.6 (C-5'), 143.3 (C-4'), 143.7 (C-7'), 153.3 (C-3), 153.4 (C-8'a), 160.3 (C-2'), 160.6 (C-6), 193.0 ($\text{O}=\text{C}-\text{Me}$). Anal. Calcd for $\text{C}_{16}\text{H}_{15}\text{N}_3\text{O}_5$ (329.31 g/mol): C 58.36, H 4.59, N 12.76, found: C 58.45, H 4.62, N 12.58. HRMS(ESI) m/z : Calcd for $\text{C}_{16}\text{H}_{16}\text{N}_3\text{O}_5$ $[\text{M} + \text{H}]^+$ 330.10900, found 330.10845.

2.4.6 (5S)-3-acetyl-5-((R)-1-hydroxyethyl)-1-(4-methyl-2-oxo-2H-chromen-7-yl)-4,5-dihydro-1,2,4-triazin-6(1H)-one (**8f**)

Yield = 0.50 g (81%), m.p. 254 – 256 °C. $[\alpha]_D^{25}$ -436 ($c \sim 1$, DMF). $^1\text{H NMR}$ (300 MHz, DMSO- d_6) δ : 1.08 (br s, 3H, CH_3 -CH), 2.40 (br s, 3H, CH_3 -4'), 2.46 (s, 3H, $\text{O}=\text{C}-\text{CH}_3$), 3.33 (s, 1H, OH), 3.89 (br s, 1H, H-5), 4.01 (br s, 1H, CH-5), 5.00 (br s, 1H, NH-4), 6.33 (d, $J = 1.0$, 1H, H-3'), 7.64 (d, $J = 2.0$ Hz, 1H, H-8'), 7.72 (dd, $J = 8.7$ Hz, 2.0, 1H, H-6'), 7.75 (d, $J = 8.7$ Hz, 1H, H-5'). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ : 18.6 (CH_3 -4'), 19.6 (CH_3 -CH), 24.8 ($\text{O}=\text{C}-\text{CH}_3$), 59.5 (CH-5), 68.6 (C-5), 111.4 (C-8'), 114.2 (C-3'), 117.7 (C-4'a), 120.0 (C-6'), 125.7 (C-5'), 143.3 (C-4'), 143.9 (C-7'), 153.3 (C-3), 153.4 (C-8'a), 160.4 (C-2'), 162.4 (C-6), 193.2 ($\text{O}=\text{C}-\text{Me}$). Anal. Calcd for $\text{C}_{17}\text{H}_{17}\text{N}_3\text{O}_5$ (343.33 g/mol): C 59.47, H 4.99, N 12.24, found: C 59.32, H 4.93, N 12.16. HRMS(ESI) m/z : Calcd for $\text{C}_{17}\text{H}_{16}\text{N}_3\text{O}_5$ $[\text{M} - \text{H}]^-$ 342.10900, found 342.10954.

2.4.7 (S)-3-Acetyl-1-(4-methyl-2-oxo-2H-chromen-7-yl)-5-(2-(methylthio)ethyl)-4,5-dihydro-1,2,4-triazin-6(1H)-one (**8g**)

Yield = 0.56 g (83%), m.p. 157 – 159 °C. $[\alpha]_D^{25}$ -344 ($c \sim 1$, DMF). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ : 2.09 (s, 3H, CH_3 S), 2.22 (m, 2H, CH_2CH), 2.43 (d, $J = 1.0$ Hz, 3H, CH_3 -4'), 2.46 (s, 3H, $\text{O}=\text{C}-\text{CH}_3$), 2.65 (t, $J = 7.0$, Hz, 2H, CH_2 S), 4.37 (m, 1H, H-5), 6.17 (s, 1H, NH-4), 6.27 (d, $J = 1.0$ Hz, 1H, H-3'), 7.60 (d, $J = 8.7$ Hz, 1H, H-5'), 7.67 (d, $J = 2.0$ Hz, 1H, H-8'), 7.71 (dd, $J = 8.7$, 2.0 Hz, 1H, H-6'). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ : 15.3 (CH_3 S), 18.7 (CH_3 -4'), 23.9 ($\text{O}=\text{C}-\text{CH}_3$), 29.7 (CH_2CH), 32.0 (CH_2 S), 52.9 (C-5), 112.0 (C-8'), 114.8 (C-3'), 118.0 (C-4'a), 119.8 (C-6'), 124.4 (C-5'), 142.0 (C-4'), 143.4 (C-7'), 151.0 (C-3), 153.6 (C-8'a), 160.8 (C-2'), 162.0 (C-6), 192.7 ($\text{O}=\text{C}-\text{Me}$). Anal. Calcd for $\text{C}_{18}\text{H}_{19}\text{N}_3\text{O}_4\text{S}$ (373.43 g/mol): C 57.89, H 5.13, N 11.25, S, 8.59, found: C 58.05, H 5.06, N 11.15, S, 8.44. HRMS(ESI) m/z : Calcd for $\text{C}_{18}\text{H}_{18}\text{N}_3\text{O}_4\text{S}$ $[\text{M} - \text{H}]^-$ 372.10180, found 372.10153.

2.4.8 (S)-Methyl-2-(3-acetyl-1-(4-methyl-2-oxo-2H-chromen-7-yl)-6-oxo-1,4,5,6-tetrahydro-1,2,4-triazin-5-yl)acetate (**8h**)

Yield = 0.44 g (66%), m.p. 150 – 152 °C. $[\alpha]_D^{25}$ -85 ($c \sim 1$, DMF). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ : 2.43 (d, $J = 1.0$ Hz, 3H, CH_3 -4'), 2.49 (s, 3H, $\text{O}=\text{C}-\text{CH}_3$), 2.85 (m, 1H, CH_2 -5), 3.13 (m, 1H, CH_2 -5), 3.74 (s, 3H, $\text{O}=\text{C}-\text{OCH}_3$), 4.60 (m, 1H, H-5), 6.27 (d, $J = 1.0$ Hz, 1H, H-3'), 6.42 (s, 1H, H-4), 7.59 (d, $J = 8.7$ Hz, 1H, H-5'), 7.62 (d, $J = 2.0$ Hz, 1H, H-8'), 7.72 (dd, $J = 8.7$, 2.0 Hz, 1H, H-6'). $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ : 18.7 (CH_3 -4'), 23.9 ($\text{O}=\text{C}-\text{CH}_3$), 37.6 (CH_2 -5), 52.4 (C-5), 112.0 (C-8'), 114.8 (C-3'), 118.2 (C-4'a), 119.8 (C-6'), 124.4 (C-5'), 141.7 (C-4'), 143.2 (C-7'), 152.0 (C-3), 153.6 (C-8'a), 160.8 (C-6), 160.9 (C-2'), 172.0 (CO_2Me), 192.8 ($\text{O}=\text{C}-\text{Me}$). Anal. Calcd for $\text{C}_{18}\text{H}_{17}\text{N}_3\text{O}_6$ (371.34 g/mol): C 58.22, H 4.61, N 11.32, found: C 57.98, H 4.55, N 11.40. HRMS(ESI) m/z : Calcd for $\text{C}_{18}\text{H}_{17}\text{N}_3\text{NaO}_6$ $[\text{M} + \text{Na}]^+$ 394.10150, found 394.10096.

2.4.9 (S)-Methyl-3-(3-acetyl-1-(4-methyl-2-oxo-2H-chromen-7-yl)-6-oxo-1,4,5,6-tetrahydro-1,2,4-triazin-5-yl)propanoate (**8i**)

Yield = 0.47 g (68%), m.p. 182 – 183 °C. $[\alpha]_D^{25}$ -184 ($c \sim 1$, DMF). $^1\text{H NMR}$ (300 MHz, CDCl_3) δ : 2.21 (br m, 2H, CH_2CH), 2.40 (br m, 2H, CH_2COOMe), 2.43 (br s, 3H, CH_3 -4'), 2.50 (s, 3H, $\text{O}=\text{C}-\text{CH}_3$), 3.68 (s, 3H, CH_3OCO), 4.29 (m, 1H, H-5), 6.14 (s, 1H, NH-4), 6.27 (br s, 1H, H-3'), 7.59 (d, $J = 8.5$ Hz, 1H, H-5'), 7.61 (br s, 1H, H-8'), 7.69 (d, $J = 8.5$ Hz, 1H, H-6'). $^{13}\text{C NMR}$ (75 MHz, DMSO- d_6) δ : 18.7 (CH_3 -4'), 23.9 ($\text{O}=\text{C}-\text{CH}_3$), 28.6 (CH_2CH), 29.7 (CH_2COOMe), 52.0 (C-5), 53.1 (CO_2CH_3), 112.0 (C-8'), 114.8 (C-3'), 118.1 (C-4'a), 119.7 (C-6'), 124.4 (C-5'), 142.2 (C-4'), 143.3 (C-7'), 152.0 (C-3), 153.6 (C-8'a), 160.8 (C-2'), 161.7 (C-6), 173.1 (CO_2Me), 192.6 ($\text{O}=\text{C}-\text{Me}$). Anal. Calcd for $\text{C}_{19}\text{H}_{19}\text{N}_3\text{O}_6$ (385.37 g/mol): C 59.22, H 4.97, N 10.90, found: C 59.08, H 5.02, N 10.81. HRMS(ESI) m/z : Calcd for $\text{C}_{19}\text{H}_{19}\text{N}_3\text{NaO}_6$ $[\text{M} + \text{Na}]^+$ 408.11716, found 408.11661.

2.4.10 (S)-5-((1*H*-Indol-3-yl)methyl)-3-acetyl-1-(4-methyl-2-oxo-2*H*-chromen-7-yl)-4,5-dihydro-1,2,4-triazin-6(1*H*)-one (**8j**)

Yield = 0.52 g (67%), m.p. 269 – 272 °C. $[\alpha]_D -422$ ($c \sim 1$, DMF). $^1\text{H NMR}$ (300 MHz, DMSO- d_6) δ : 1.89 (s, 3H, O=C-CH₃), 2.37 (d, $J = 1.0$ Hz, 3H, CH₃-4'), 3.16 (dd, 1H, CH₂-5), 3.32 (s, 1H, NH-4), 4.44 (br s, 1H, H-5), 6.33 (d, $J = 1.0$ Hz, 1H, H-3'), 6.82 (m, 1H, H-6''), 6.97 (m, 1H, H-7''), 7.00 (m, 1H, H-5''), 7.20 (d, $J = 2.0$ Hz, 1H, H-8'), 7.30 (dd, $J = 8.7, 2.0$ Hz, 1H, H-6'), 7.44 (d, $J = 7.9$ Hz, 1H, H-5''), 7.66 (d, $J = 8.7$ Hz, 1H, H-5'), 7.89 (s, 1H, H-2''), 10.88 (br s, 1H, NH-1''). $^{13}\text{C NMR}$ (75 MHz, DMSO- d_6): $\delta = 18.6$ (CH₃-4'), 24.2 (O=C-CH₃), 30.2 (CH₂-5), 55.1 (C-5), 108.5 (C-3''), 111.6 (C-7''), 111.7 (C-8'), 114.3 (C-3'), 117.8 (C-4'a), 118.8 (C-6'), 119.0 (C-5''), 120.3 (C-4''), 121.3 (C-6''), 124.7 (C-5'), 125.5 (C-2''), 128.2 (C-3'a), 136.5 (C-7'a), 143.3 (C-4'), 143.6 (C-7'), 153.1 (C-3), 153.4 (C-8'a), 160.3 (C-2'), 162.8 (C-6), 193.0 (O=C-Me). Anal. Calcd for C₂₄H₂₀N₄O₄ (428.44 g/mol): C 67.28, H 4.71, N 13.08, found: C 67.14, H 4.63, N 13.12. HRMS(ESI) m/z : Calcd for C₂₄H₂₀N₄NaO₄ [M + Na]⁺ 451.12522, found 451.12768.

2.4.11 3-Acetyl-4-methyl-1-(4-methyl-2-oxo-2*H*-chromen-7-yl)-4,5-dihydro-1,2,4-triazin-6(1*H*)-one (**8k**)

Yield = 0.30 g (53%), m.p. 213 – 215 °C. $^1\text{H NMR}$ (300 MHz, CDCl₃) δ : 2.43 (d, $J = 1.0$ Hz, 3H, CH₃-4'), 2.50 (s, 3H, O=C-CH₃), 3.16 (s, 3H, CH₃-(N)4), 3.99 (s, 2H, H₂-5), 6.26 (d, $J = 1.0$ Hz, 1H, H-3'), 7.59 (d, $J = 8.7$ Hz, 1H, H-5'), 7.72 (d, $J = 2.0$ Hz, 1H, H-8'), 7.77 (dd, $J = 8.7, 2.0$ Hz, 1H, H-6'). $^{13}\text{C NMR}$ (75 MHz, CDCl₃) δ : 18.7 (CH₃-4'), 27.7 (O=C-CH₃), 38.4 (CH₃-(N)4), 52.4 (C-5), 111.9 (C-8'), 114.6 (C-3'), 118.0 (C-4'a), 119.4 (C-6'), 124.4 (C-5'), 142.9 (C-4'), 144.0 (C-7'), 152.0 (C-3), 153.7 (C-8'a), 159.3 (C-6), 160.9 (C-2'), 192.8 (O=C-Me). Anal. Calcd for C₁₆H₁₅N₃O₄ (313.31 g/mol): C 61.34, H 4.83, N 13.41, found: C 61.22, H 4.80, N 13.34. HRMS(ESI) m/z : Calcd for C₁₆H₁₅N₃NaO₄ [M + Na]⁺ 336.09603, found 336.09548.

2.4.12 (S)-4-Acetyl-2-(4-methyl-2-oxo-2*H*-chromen-7-yl)-6,7,8a-tetrahydro-pyrrolo[1,2-*d*][1,2,4]-triazin-1(2*H*)-one (**12**)

Yield = 0.21 g (34%), m.p. 183 – 185 °C. $[\alpha]_D +550$ ($c \sim 1$, DMF). $^1\text{H NMR}$ (300 MHz, CDCl₃) δ : 1.95 (m, 2H, H₂-6), 2.31 (m, 2H, H₂-7), 2.42 (d, $J = 1.0$ Hz, 3H, CH₃-4'), 2.50 (s, 3H, O=C-CH₃), 3.82 (m, 1H, H-7a), 3.97 (m, 2H, H₂-5), 6.24 (d, $J = 1.0$ Hz, 1H, H-3'), 7.58 (d, $J = 8.7$ Hz, 1H, H-5'), 7.70 (d, $J = 2.0$ Hz, 1H, H-8'), 7.75 (dd, $J = 8.7, 2.0$ Hz, 1H, H-6'). $^{13}\text{C NMR}$ (75 MHz, CDCl₃) δ : 18.8 (CH₃-4'), 24.2 (C-6), 26.3 (O=C-CH₃), 27.4 (C-7), 50.0 (C-5), 57.9 (C-7a), 111.6 (C-8'), 114.5 (C-3'), 117.8 (C-4'a), 119.4 (C-6'), 124.4 (C-5'), 143.3 (C-4'), 143.8 (C-7'), 152.1 (C-4), 153.6 (C-8'a), 161.0 (C-2'), 161.8 (C-1), 194.4 (O=C-Me). Anal. Calcd for C₁₈H₁₇N₃O₄ (339.35 g/mol): C 63.71, H 5.05, N 12.38, found: C 63.58, H 4.97, N 12.23. HRMS(ESI) m/z : Calcd for C₁₈H₁₇N₃NaO₄ [M + Na]⁺ 362.11168, found 362.11113.

2.5 Cell culture

A validated and published cell culture method was used for the study (Agu et al., 2009). Human nasal epithelial tissues were obtained during elective surgeries from 2 patients. Use of the biopsies was approved by QEII Regional Hospital Research Ethics Board (REB # CDHA-RS/2006-352). The tissues were transported in DMEM-F12 1/1 culture medium supplemented with streptomycin 100 $\mu\text{g/mL}$ and penicillin 100 IU/mL. The medium for the monolayer culture was Ultrosor G 2.0% in DMEM-F12 1/1 supplemented with, streptomycin 100 $\mu\text{g/mL}$ and penicillin 100 IU/mL.

The human nasal epithelial tissues obtained during surgery were washed three times with physiological saline solution supplemented with antibiotics. The cells were dissociated enzymatically for a period of 16 – 24 hours at 4 °C using 0.1% pronase. The pronase was deactivated with 10% NU-serum prior to cell washing with DMEM-F12 1/1. The washing solution was removed after centrifugation at 70 g for 5 minutes on each occasion. The resulting suspension of cells was pre-plated on plastic for 1 hour at 37 °C in a 95% O₂ and 5% CO₂ environment to reduce fibroblast contamination. Subsequently, the cells were seeded at a density of 5.0×10^5 cells/well. The cells were incubated at 37 °C in a 95% O₂ and 5% CO₂ environment using DMEM F12 supplemented with Ultrosor G 2%. The medium was changed every 2 days till used for experiment (7 – 14 days).

2.6 Preparation of nasal homogenates

The human nasal epithelial tissues and cultured cells were homogenized in Hanks Balanced salt buffer with a tissue homogenizer (PowerGen 125, Fisher scientific, Ottawa, ON, Canada) at 2500 rpm. The homogenized cells were centrifuged at 10000 x g for 10 min at 4 °C to remove cellular and nuclear debris. The supernatant was used for protein assay using the bicinchoninic acid assay method and for the determination of aminopeptidase activity. The supernatant was kept on ice and used within 4 hours of preparation.

2.7 Assay for aminopeptidase activity

The method described in our recent paper (Agu et al., 2009) was used to determine the aminopeptidase activity of the cell culture homogenates using Ala-MCA (control) and the synthesized compounds (0.125, 0.5 mM). The homogenate samples (50 µg protein in 950 µL PBS) were pre-incubated at 37 °C for 2 min. Metabolism was initiated by the addition of 50 µL (0.125 or 0.5 mM) of the substrate and incubated for 10 min at 37 °C. The reaction was terminated by adding 2 mL of stop buffer (0.12 M Monochloroacetic acid and 0.13 M sodium acetate, pH 4.3) and 7-amino-4-methylcoumarin formed from the substrates was measured using a Modulus single tube multimode fluorescence reader (model 9200, Turner Bio systems CA, USA) with a UV fluorescent optical kit module (model # 9200-041). Aminopeptidase activity was expressed in fluorescent units (FSU). Differences between the aminopeptidase activity of the cells incubated with synthesized substrates and control (Ala-MCA) were determined by analysis of variance (ANOVA). Acceptable level of significance was set at $p < 0.05$.

2.8 RNA isolation and cDNA synthesis

Total RNA was extracted from the nasal epithelial cells using TriZol according to the manufacturer's instructions. In brief, cells were lysed with Trizol 1 mL per 10 cm². Subsequently, 200 µL of chloroform was added per 1 mL of TriZol and vortexed. Three phases were separated by centrifuging for 15 minutes at 13,000 rpm at 4 °C. Only the colorless upper aqueous RNA phase was removed and vortexed with isopropanol to precipitate the RNA. Samples were incubated at room temperature for 10 minutes before centrifugation for 10 minutes at 13000 rpm at 4 °C. The resulting RNA pellet was washed twice with ice cold 75% ethanol and then re-suspended in ddH₂O. Concentration and purity of RNA was measured using GeneQuant Pro®. All samples had A₂₆₀/A₂₈₀ absorbance readings greater than 1.6 confirming high RNA purity. Two micrograms of isolated RNA was transcribed into cDNA by M-MuLV reverse transcriptase using 5 mM dNTP and 1 µL of oligo dT primers and 2 µL of cDNA buffer.

2.9 Polymerase chain reaction (PCR)

Polymerase chain reaction was carried out in a solution containing 1.5 µL 50 µM MgCl₂, 2.5 µL Taq PCR buffer, 1 µL 5 mM dNTP, 1 µL of 10 mM forward and reverse primer, 2 µL of cDNA, 0.5 units of Taq polymerase and water up to 25 µL. Gene sequences for primer design were obtained from the National Center for Biotechnology Information's GenBank. Primers were designed using OligoPerfect™ Designer from Invitrogen. APN (ANPEP) forward and reverse primer sequences as follows: forward 5'-CCTCAATGTGACGGGCTATT -3' (corresponding to bases 2161 – 2180) reverse 5'- TCATTGACCAGTGTGGCATT -3' (corresponding to bases 2763 – 2744). PCR product was 603-bp. APB (RNPEP) forward and reverse primer sequences were as follows: forward 5'- GAGCCCGTGAGCTTCTACAC -3' (corresponding to bases 345 – 364) reverse 5'- GTGGCATGAAGAGCAAGTCA -3' (corresponding to bases 906 – 877). PCR product was 562-bp. DPPIV forward and reverse primer sequences were as follows: forward 5'-CAAATTGAAGCAGCCAGACA-3' (corresponding to bases 2377 – 2396) reverse 5'- TGAAGTGGCTCATGTGGGTA -3' (corresponding to bases 2836 – 2817). The PCR product was 460-bp. The solution experienced an initial denaturation at 94 °C for 5 minutes and then 35 cycles of denaturing at 94 °C for 30 seconds, annealing at 55 °C for 45 seconds, and extension at 72 °C for 1 minute. A final extension was applied for 10 minutes at 72 °C. A negative control was done using RNA and APN primers only. Amplified DNA was electrophoresed on a 1% agarose gel containing ethidium bromide.

3. Results and discussion

3.1 Synthesis

The hydrazonoyl chloride synthon (**10**), required in this study, was prepared *via* direct coupling of 7-coumarin diazonium chloride with 3-chloropentane-2,4-dione (Japp-Klingemann reaction) (Scheme 1) (Phillips, 1959; Yao & Resnick, 1962; Barrett et al., 1970).

The intermediate arene diazonium chloride solution was freshly prepared by diazotization of 7-amino-4-methylcoumarin which, in turn, was prepared from *m*-aminophenol according to literature procedure (Pozdnev, 1990). α -Amino esters (**11a-k**), acting as nitrogen nucleophiles, are expected to add onto nitrile imine, generated *in situ* from the corresponding hydrazonoyl chloride (**10**) by the action of triethyl amine, to give initially the corresponding *Z*-amidrazone ester as the kinetically controlled product, the latter transient acyclic adducts undergo spontaneous intramolecular condensation, involving the amidrazone nitrogen and the ester carbonyl group, to yield the respective targeted heterocycles (**8a-k**) (Scheme 2).

Proline methyl ester reacted with (**10**) in a similar fashion to give the corresponding bicyclic triazinone (**12**) (Scheme 3). This methodology was modeled on previously reported synthesis of related N-aryl 4,5-dihydro-1,2,4-triazin-2-ones *via* reacting α -amino esters with N-arylhydrazonoyl chlorides (El-Abadelah et al., 1991).

The newly synthesized compounds **10**, **8a-k** and **12** were characterized by elemental analyses, MS and NMR spectral data. These data, detailed in the experimental part, are consistent with the suggested structures. Thus, the mass spectra display the correct molecular ion peaks for which the measured high resolution (HRMS) data are in good agreement with the calculated values. DEPT and 2D (COSY, HMQC, HMBC) experiments showed correlations that helped in the ^1H - and ^{13}C -signal assignments to the different carbons and their attached, and/or neighboring hydrogens.

3.2 Pharmacological activity

Studies that involved the pharmacological activity of the synthesized compounds elucidated the conversion of the test compounds (N-(coumarin-7-yl)-4,5-dihydro-1,2,4-triazin-6(1*H*)-ones) to 7-amino-4-methylcoumarin by aminopeptidases of the human nasal epithelium. This tissue culture model was chosen based on our earlier work that demonstrated the expression of various aminopeptidases in cultured human nasal epithelium (Agu et al., 2009). Prior to investigating the pharmacological activities of the synthesized substrates, we conducted an in-house validation of the cells to demonstrate the expression of three types of aminopeptidases (APN, APB and DPPIV) in the cell batches that we used for the studies (Fig. 4).

As expected, gene transcripts for aminopeptidase N (600 bp), aminopeptidase B (~ 550 bp) and DPPIV (~ 500 bp) were detected. Control reactions with water did not yield any gene transcript. This implied that the gene transcripts that we observed were from aminopeptidase. Apart from genetic demonstration of the expression of the enzymes in the nasal tissues, it was important to show the ability of the synthesized compounds to act as substrates for these enzymes. In a previous study, we demonstrated that 7-amino-4-methylcoumarin can be formed from specific substrates for APN (L-alanine-4-methylcoumaryl-7-amide, APB (L-arginine-4-methylcoumaryl-7-amide) and DPPIV (glycyl-L-proline-4-methylcoumaryl-7-amide) (Agu et al., 2009). In this study we chose L-alanine-4-methylcoumaryl-7-amide as our model substrate because of the intensity of the gene transcript that we observed during the in-house validation. Furthermore, the time-concentration profile for 7-amino-4-methylcoumarin formation from Ala-AMC was linear within the time and concentration range that we investigated and hence making the compound a good reference for the synthesized compounds. Fig. 5 shows both time and concentration-dependent release of 7-amino-4-methylcoumarin from Ala-MCA. At 0.5 mM of the substrate, up to 80,000 FSU of 7-amino-4-methylcoumarin was released in 1 hr. This quantity was significantly higher than about 20,000 FSU that was released following the incubation of the cells with 0.125 mM of the substrate (Ala-MCA). At the concentrations that were investigated (0.125, 0.5 mM), the yield of 7-amino-4-methylcoumarin was linear.

Fig. 6 shows the release of 7-amino-4-methylcoumarin from compounds **8a** and **8c**. Although no clear concentration-dependency was observed for compound **8a**, higher concentration of 7-amino-4-methylcoumarin was released for compound **8c** at 0.5 mM compared to 0.125 mM. When compared to the control (Ala-MCA), the quantity of 7-amino-4-methylcoumarin that was formed from both compounds (**8a** and **8c**) was significantly lower. As was observed in Fig. 6, the formation of 7-amino-4-methylcoumarin for compounds **8e** and **8f** was comparable to compounds **8a** and **8c** (Fig. 7).

The fluorescence associated with the metabolite for both **8e** and **8f** was significantly lower than that generated by Ala-MCA breakdown at equimolar concentrations and same exposure time ($p > 0.05$). At 0.125 and 0.5 Mm, both **8g** and **8h** result in 7-amino-4-methylcoumarin fluoresce of less than 5000 FSU (Fig. 8).

This implies that the fluorescence associated with both compounds at the tested concentrations and incubation period were significantly lower than that of Ala-MCA. In a similar manner, the fluorescent yield corresponding to **8i** and **8k** metabolic breakdown were statistically lower than the Ala-MCA fluorescent intensity ($p > 0.05$) (Fig. 9).

4. Conclusion

Over all, the metabolic cleavage of (N-(coumarin-7-yl)-4,5-dihydro-1,2,4-triazin-6(1*H*)-ones) to 7-amino-methylcoumarin was observed. However, a comparison of the metabolic breakdown of the synthesized substrates in the human nasal culture model relative to Ala-MCA indicated that the latter is a better substrate for aminopeptidases than our compounds. The disparity in the yield of 7-amino-4-methylcoumarin between our series of compounds and the model substrate could be related to the unmasked α -amino acid residues in the

model substrate. In our compounds, the masking of the α -amino acid, a strategy that was intended to make the compounds metabolically stealth for potential in vivo applications could be responsible for preventing the hydrolysis of the amide bonds to liberate fluorescent AMC.

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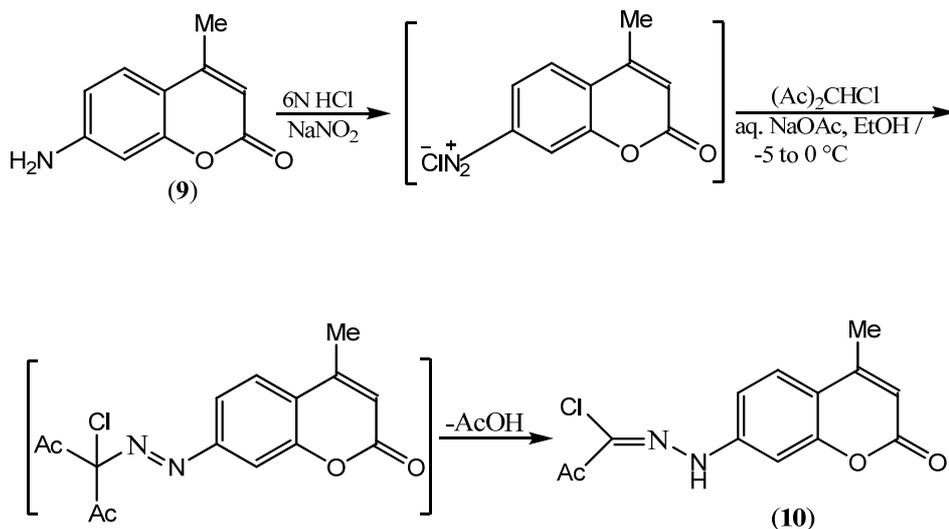
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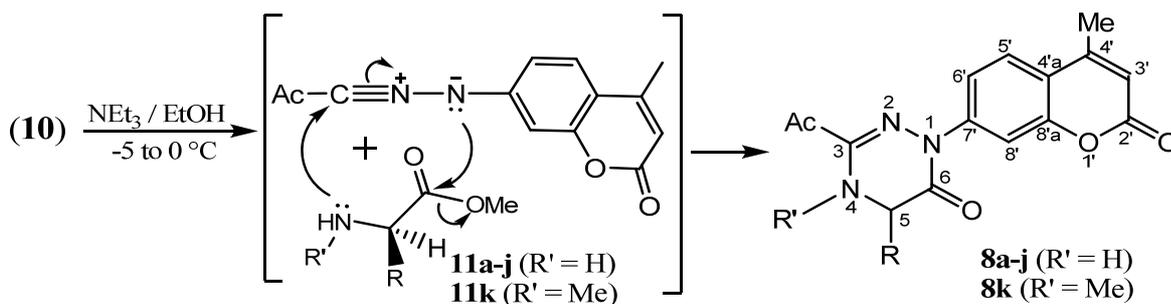
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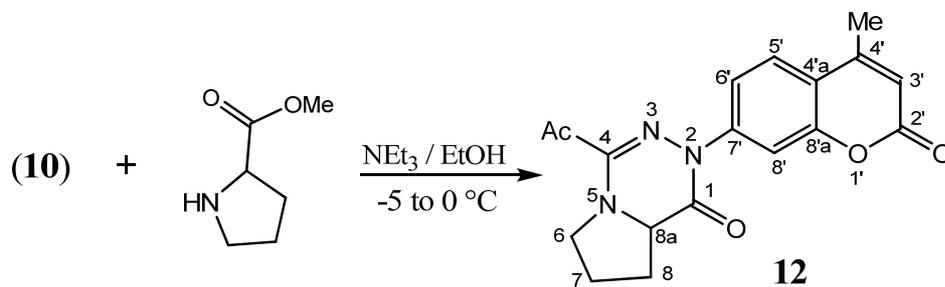
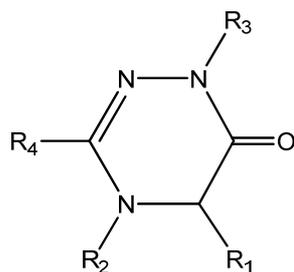


Scheme 1. Synthesis of N-(4-methyl-2-oxo-2H-chromen-7-yl)-2-oxopropanehydrazonoyl chloride (**10**)



entry	R	entry	R	entry	R
a	H	e	CH ₂ OH	i	CH ₂ CH ₂ CO ₂ Me
b	Me	f	CH(OH)Me	j	
c	CH ₂ CHMe ₂	g	CH ₂ CH ₂ SMe	k	H
d	CH ₂ Ph	h	CH ₂ CO ₂ Me		

Scheme 2. Synthesis of compounds (**8a-k**)

Scheme 3. Synthesis of pyrrolo[1,2-d][1,2,4]triazin-1-one (**12**)

1 (R_1 = amino acid portion;

R_2 = H, Me, Bz;

R_3 = substituted phenyl;

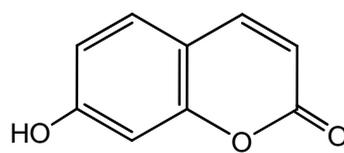
R_4 = Ac, PhCO, CO₂Et)

2 (R_4 = PhNHCO; R_1 - R_3 : as in **1** above)

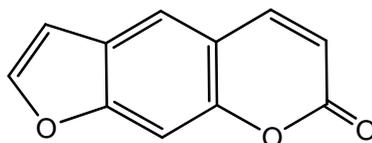
3 (R_1 = R_2 = H; R_3 = Me; R_4 = PhCO)

4 (R_2 = NH₂; R_1 , R_3 or R_4 : as in **1** above)

Figure 1. Some synthetic 4,5-dihydro-1,2,4-triazin-6-ones



(**5**)



(**6**)

Figure 2. Some naturally occurring coumarins

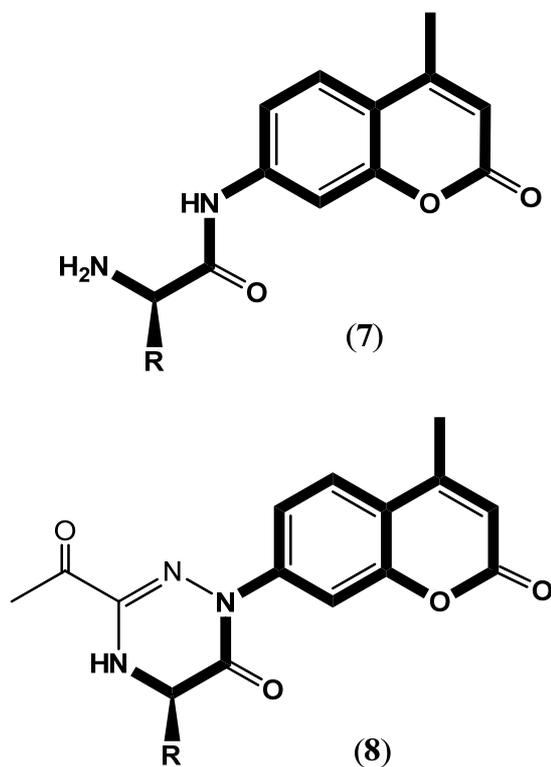


Figure 3. AA-AMC's (7) and masked AA-AMC's (8)

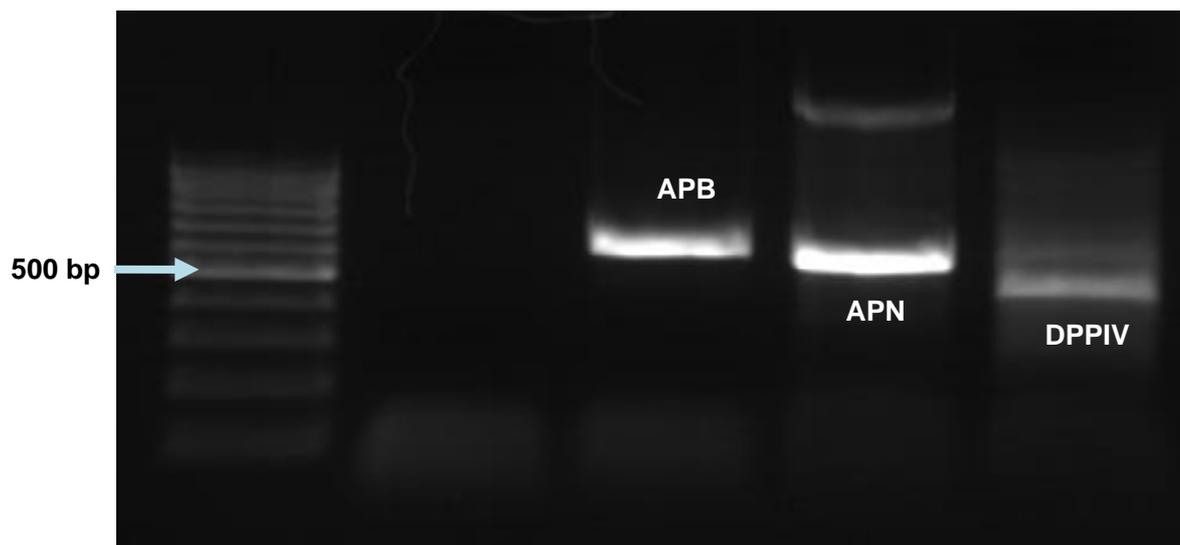


Figure 4. Gene transcripts for aminopeptidase B (APB), aminopeptidase N (APN) and dipeptidyl dipeptidases (DPPIV) in human nasal cell homogenates

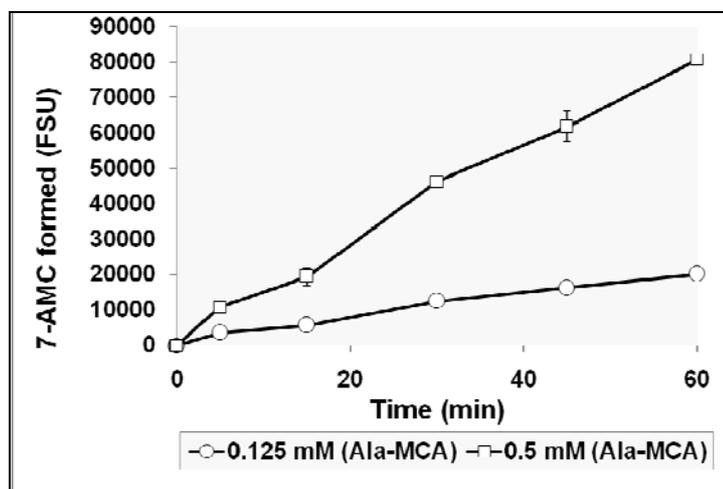


Figure 5. Time-dependent release of 7-amino-4-methylcoumarin from L-alanine-4-methyl-coumaryl-7-amide (positive control) in primary human nasal cell homogenates. Each point represents the mean \pm SD, n = 2

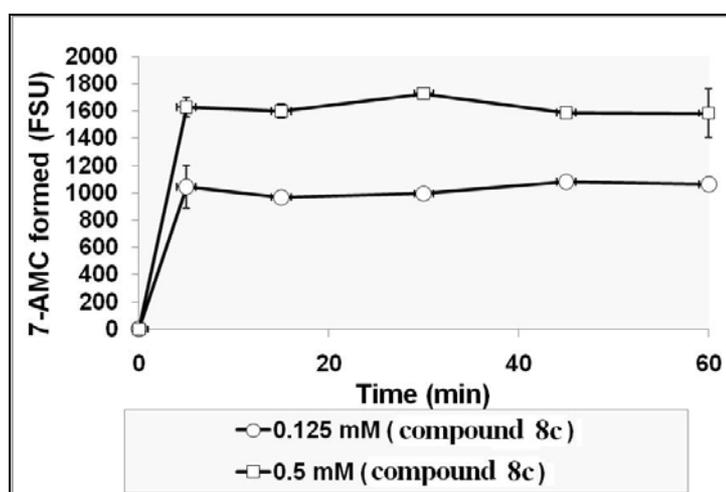
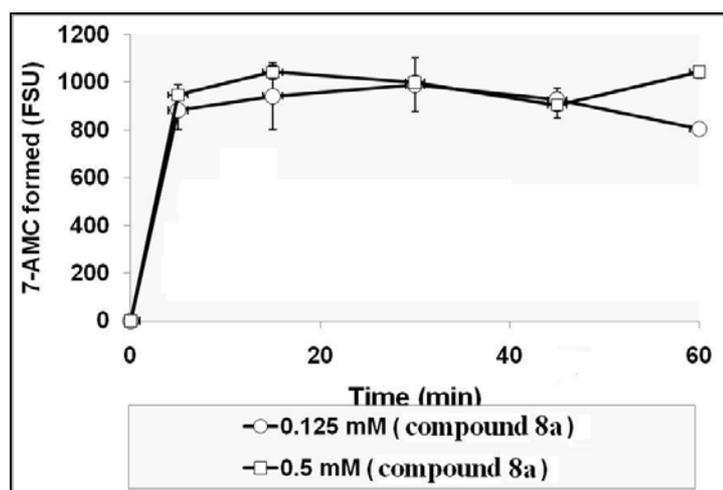


Figure 6. Time-dependent release of 7-amino-4-methylcoumarin from compounds **8a** and **8c** in primary human nasal cell homogenates. Each point represents the mean \pm SD, n = 2

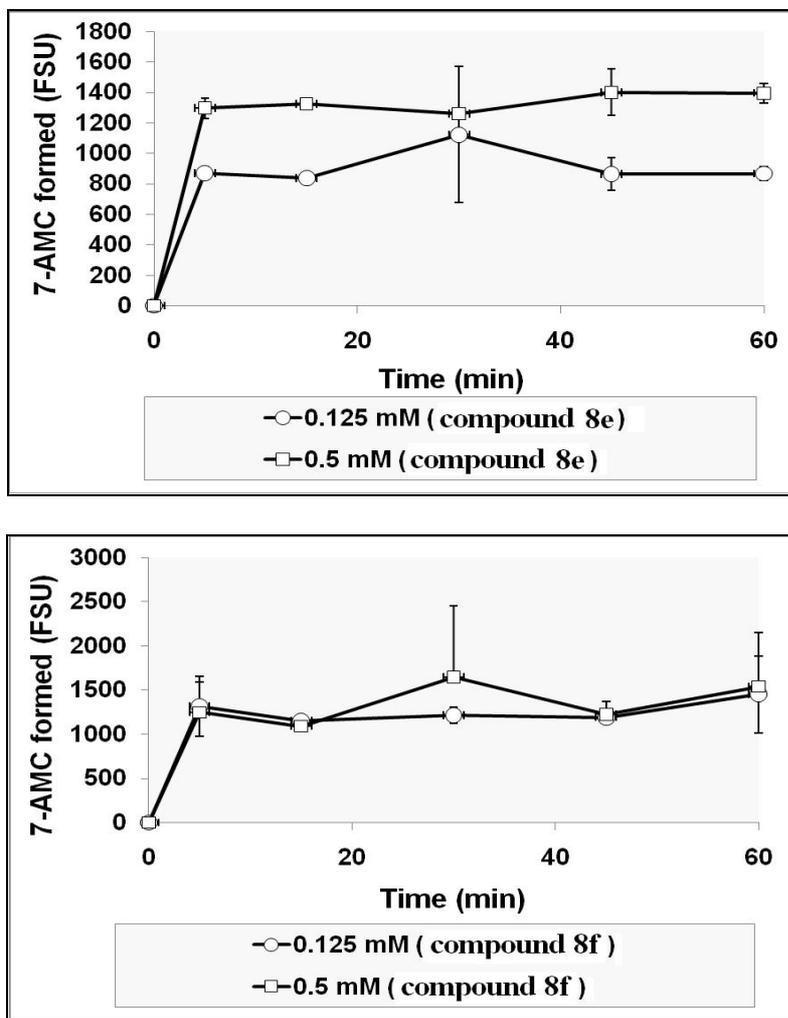
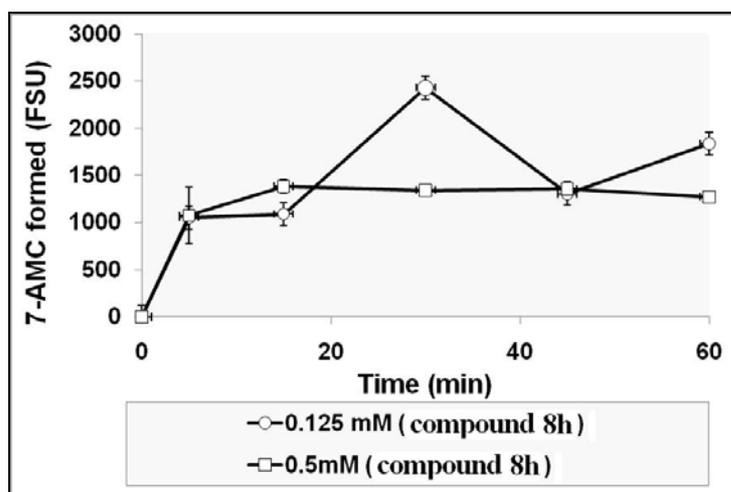


Figure 7. Time-dependent release of 7-amino-4-methylcoumarin from compounds **8e** and **8f** in primary human nasal cell homogenates. Each point represents the mean \pm SD, n = 2



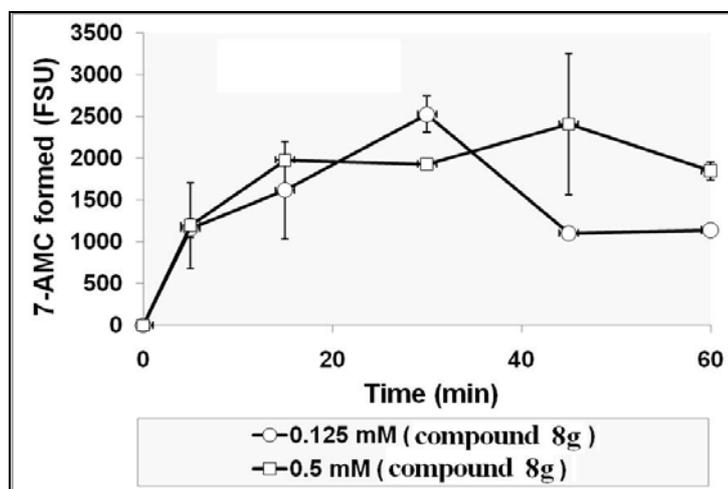


Figure 8. Time-dependent release of 7-amino-4-methylcoumarin from compounds **8g** and **8h** in primary human nasal cell homogenates. Each point represents the mean \pm SD, n = 2

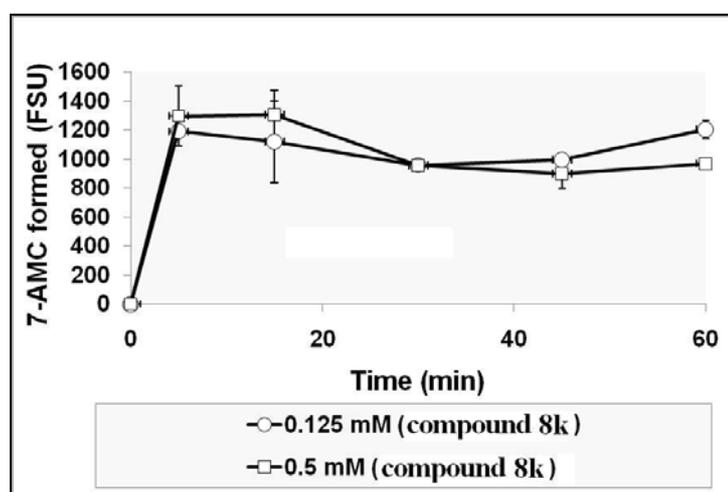
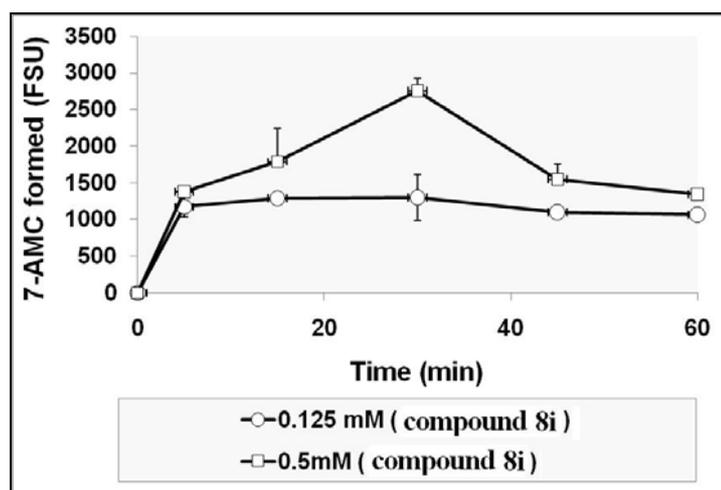


Figure 9. Time-dependent release of 7-amino-4-methylcoumarin from compounds **8i** and **8k** in primary human nasal cell homogenates. Each point represents the mean \pm SD, n = 2